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# Hemolytic and Sphingomyelinase Activities of *Clostridium perfringens* Alpha-Toxin Are Dependent on a Domain Homologous to That of an Enzyme from the Human Arachidonic Acid Pathway

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**The N-terminal domain of *Clostridium perfringens* alpha-toxin, homologous with the nontoxic phospholipase C of *Bacillus cereus*, was expressed in *Escherichia coli* and shown to retain all of the phosphatidylcholine hydrolyzing activity of the alpha-toxin, but not the sphingomyelinase, hemolytic, or lethal activities. The C-terminal domain of alpha-toxin showed sequence and predicted structural homologies with the N-terminal region of arachidonate 5-lipoxygenase, an enzyme from the human arachidonic acid pathway which plays a role in inflammatory and cardiovascular diseases in humans.**

The alpha-toxin of *Clostridium perfringens* (CPA) was the first bacterial protein shown to possess both enzymatic activity (phospholipase C) and toxic properties (10). The single polypeptide toxin is considered to be the major toxin of biotype A strains (12, 21), which are often associated with gas gangrene infections in humans, and it has been suggested that CPA plays an important role in the pathogenesis of disease (6, 21). Some experimental evidence supports this hypothesis; the toxin is myonecrotic and the tissue damage associated with gas gangrene can be partially reproduced on administration of the toxin to animals (6). Also, prior immunization with a crude toxoid vaccine has been shown to protect mice from *C. perfringens* infection (6).

In contrast to CPA, the phospholipase C of *Bacillus cereus* is considered to be nontoxic (13). The toxicity of some phospholipase C's may be attributable to their ability to interact with, and degrade, not only phosphatidylcholine but also sphingomyelin, which are key components of eukaryotic cell membranes (13). This property can be determined in vitro by measuring the hemolysis of erythrocytes (13, 21). Sublytic concentrations of CPA can cause the contraction of smooth muscle (1, 2), thromboxane release from cells (2), and blood platelet aggregation (23) and can elicit cardiovascular (22) and other effects which may contribute to the pathogenesis of gas gangrene.

The N-terminal two-thirds of the deduced amino acid sequence of CPA is approximately 29% homologous (9, 16, 24, 26) with the non-toxic (13) *B. cereus* phosphatidylcholine-preferring phospholipase C (PC-PLC), and many functionally important residues are conserved (4, 9, 25). As CPA and PC-PLC share many properties, including thermostability (7, 15) and the requirement for catalytically essential zinc ions (4, 7), it is possible that this region of CPA is responsible for phospholipase C activity (9, 16, 24, 26). We have constructed a plasmid (pB15b) containing a DNA fragment predicted to encode the region of CPA (residues 1 to 249 [$CPA_{249}$]) homologous with PC-PLC and seven additional amino acids, encoded by the pUC18 vector, before termination (Fig. 1). $CPA_{249}$, purified from 12-h cultures by the method described previously for CPA (24), purified *Escherichia coli*-cloned CPA (24), and PC-PLC (type XIII; Sigma) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting), using anti-CPA primary monoclonal antibody 3α4D10 (20, 24). The immunological identity of $CPA_{249}$ was confirmed, and its molecular weight was estimated to be 27,700 (predicted, 29,389). PC-PLC did not react with 3α4D10.

The specific activities of purified CPA and $CPA_{249}$ against egg yolk substrate were similar, but $CPA_{249}$ was approximately 4.5 times more active against the synthetic substrate, *p*-nitrophenol phosphorylcholine. PC-PLC hydrolyzed both substrates more rapidly than either CPA or $CPA_{249}$ (Table 1). Only CPA caused hemolysis of mouse erythrocytes (Table 1). Similarly, $CPA_{249}$ and PC-PLC were nontoxic for mice at doses of 10 μg/mouse, while CPA caused five deaths ($n = 6$) after inoculation of 1 μg of protein.

The ability of the each enzyme (10 μg) to hydrolyze purified phosphatidylcholine (from egg yolk) or purified sphingomyelin (from bovine brain) (both at 0.25 mg/ml) after incubation for 2 h at 37°C was assessed by thin-layer chromatography (16). CPA digested both phospholipids but $CPA_{249}$ and PC-PLC hydrolyzed phosphatidylcholine much more efficiently than sphingomyelin. No hydrolysis was detected with periplasmic extracts from *E. coli* containing pUC18 (Fig. 2).

It has been suggested that both phosphatidylcholine and sphingomyelin hydrolyzing activities are necessary for a bacterial phospholipase C to be hemolytic and lethal (13). Other workers have used a panel of neutralizing monoclonal antibodies (19) or chemical modification of the toxin (17) to show that the phosphatidylcholine hydrolyzing activity of CPA was partially independent of other biological activities. In the related bacterium *B. cereus*, phosphatidylcholine and sphingomyelin hydrolyzing activities are associated with separate proteins (5, 27) which can act synergistically to cause hemolysis (3). The genes encoding these enzymes are tandemly located and coregulated (3), leading some workers to speculate that the alpha-toxin gene represents a fusion of these two elements (3, 16). However, neither the nucleotide nor the predicted amino acid sequences of the C-terminal third of CPA and *B. cereus* sphingomyelinase show significant sequence homology. Consequently, it was decided to investigate amino acid sequence homologies between the C-terminal portion of the protein (removed in $PLC_{249}$) and

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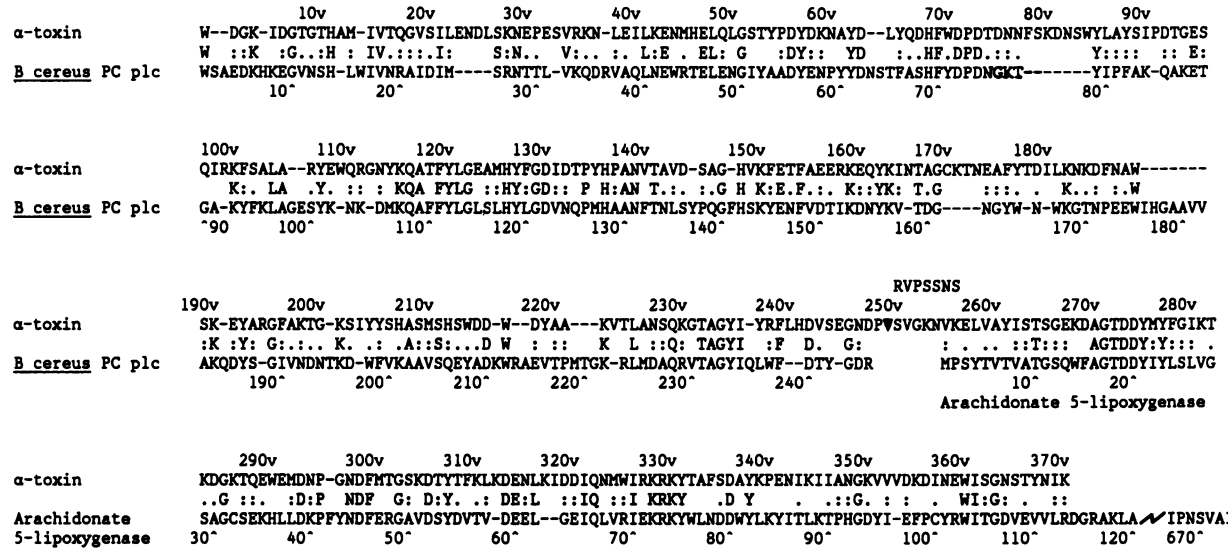


FIG. 1. Alignment of CPA (24), *B. cereus* PC-PLC (5), and HA5L (11). The middle line shows identical residues and frequently (double points) or rarely (single point) substituted amino acids. Plasmid pB15b encoding $CPA_{249}$ was constructed by ligating the products of a partial *Sau3A* digest of the 3.1-kb insert of pT5C100 (24) into suitably digested pUC18 vector plasmid. The point of truncation of the alpha-toxin is shown by an arrowhead with additional pUC18 vector-encoded amino acids found in $CPA_{249}$.

protein sequences in the PIR (Los Alamos National Laboratory) data base. We found a significant degree of sequence homology (29% identity) between residues 255 to 370 of CPA and residues 1 to 113 of a lipid-modifying enzyme, human arachidonate 5-lipoxygenase (HA5L) (11) (Fig. 1). Comparison of hydropathy profiles (Fig. 3) suggested that the C-terminal domain of CPA may be structurally similar to the homologous N-terminal region of HA5L. Five of the seven tyrosine residues in the C-terminal domain of the alpha-toxin are aligned with tyrosines in HA5L. Tyrosine residues have been identified as essential for hemolytic, platelet-aggregating, and lethal activities of CPA (17).

Our results, and the lack of homology between the CPA C-terminal domain and *B. cereus* sphingomyelinase, suggest that this domain is unlikely to exhibit sphingomyelinase activity. Rather, it seems likely that phosphatidylcholine and sphingomyelin hydrolyses both occur at the same site in CPA. With *B. cereus* PC-PLC it was shown that the substrate range could be artificially enhanced to include sphingomyelin by substituting cobalt for zinc ions in the native protein (14). This was attributed to subtle alteration of active-site architecture.

HA5L is required for the generation of leukotrienes from arachidonic acid, a phospholipid catabolite (18). Why a domain from such an enzyme confers toxicity on a bacterial phospholipase is not clear. Possibly, the N-terminal region of HA5L and the C-terminal domain of CPA are involved in recognition of structural components common to arachidonic acid and sphingomyelin. The requirement by CPA, HA5L, and *B. cereus* sphingomyelinase, but not PC-PLC, for calcium ions for substrate binding supports this (13, 27, 28). Alternatively, the C-terminal domain may distort the active site, permitting sphingomyelin hydrolysis.

These results demonstrate that a potent bacterial toxin is composed of a domain representing a functional homolog of

TABLE 1. Specific activities of CPA

| Sample[a] | Egg yolk phospholipid hydrolyzing activity (U/min/mg)[b] | pNPPC (U/mg)[c] | Hemolytic activity (U/min/mg)[d] | Lethality ($LD_{50}$ [μg]/mouse)[e] |
|---|---|---|---|---|
| Alpha-toxin | 52.3 | 6.4 | 236 | <1 |
| Alpha-truncate | 43.4 | 29 | 0 | >10 |
| PC-PLC | 1,917 | 57 | 0 | >10 |
| *E. coli* control | 0 | <0.1 | 0 | Not tested |

[a] Purified CPA, $CPA_{249}$, *B. cereus* PC-PLC, or a crude periplasmic extract from *E. coli* containing pUC18.

[b] Egg yolk phospholipid hydrolyzing activity; units as defined previously (24).

[c] *p*-Nitrophenol phosphorylcholine (pNPPC) hydrolyzing activity (25); 1 U catalyzes the hydrolysis of 1 nmol of substrate in 1 min.

[d] Hemolytic activity against 5% (vol/vol) mouse erythrocytes; units as defined previously (24).

[e] Intraperitoneal inoculation into a group ($n$ = 6) of 23-week-old BALB/c mice (18 to 22 g). $LD_{50}$, 50% lethal dose.

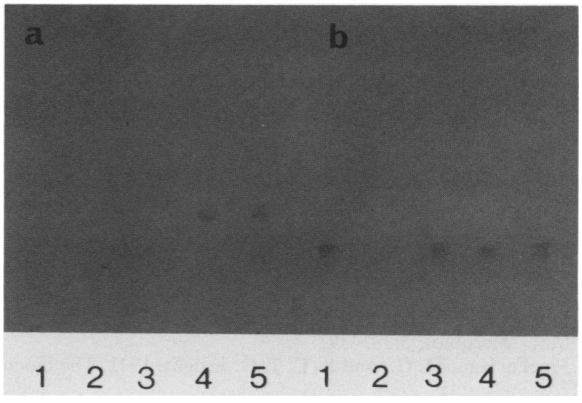


FIG. 2. Hydrolysis of phosphatidylcholine (a) or sphingomyelin (b) by purified *B. cereus* PC-PLC (lane 1), CPA (lane 2), $CPA_{249}$ (lane 3), or a crude periplasmic extract from *E. coli* containing the vector plasmid pUC18 (lane 4). Lane 5 contained only phospholipid. Remaining phospholipids were separated by thin-layer chromatography.

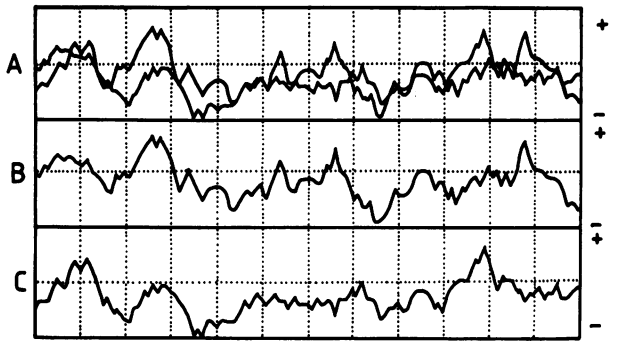


FIG. 3. Superimposed (A) and individual hydropathy profiles of residues 1 to 120 of HA5L (B) and residues 250 to 370 of CPA (C) calculated by the method of Kyte and Doolittle (8) (nine-residue window).

a nontoxic prokaryotic enzyme and a domain sharing homology with a eukaryotic enzyme which, although inherently nontoxic, catalyzes the formation of mediators of some human cardiovascular and inflammatory diseases (18). Further analysis of the C-terminal domain of CPA should also contribute to the elucidation of structure-function relationships in HA5L.

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